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From classical to current: analyzing peripheral nervous system and spinal cord lineage and fate

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Abstract

During vertebrate development, the central (CNS) and peripheral nervous systems (PNS) arise from the neural plate. Cells at the margin of the neural plate give rise to neural crest cells, which migrate extensively throughout the embryo, contributing to the majority of neurons and all of the glia of the PNS. The rest of the neural plate invaginates to form the neural tube, which expands to form the brain and spinal cord. The emergence of molecular cloning techniques and identification of fluorophores like Green Fluorescent Protein (GFP), together with transgenic and electroporation technologies, have made it possible to easily visualize the cellular and molecular events in play during nervous system formation. These lineage-tracing techniques have precisely demonstrated the migratory pathways followed by neural crest cells and increased knowledge about their differentiation into PNS derivatives. Similarly, in the spinal cord, lineage-tracing techniques have led to a greater understanding of the regional organization of multiple classes of neural progenitor and post-mitotic neurons along the different axes of the spinal cord and how these distinct classes of neurons assemble into the specific neural circuits required to realize their various functions. Here, we review how both classical and modern lineage and marker analyses have expanded our knowledge of early peripheral nervous system and spinal cord development.

Introduction

The advent of molecular cloning techniques in the early 1980s has led to a “golden age” in developmental biology. Many genes that establish the vertebrate body plan have been identified, frequently by cloning the vertebrate homologues of genes first identified in invertebrates (Nusslein-Volhard and Wieschaus, 1980). The expression patterns of these genes are often extremely informative about their function, and can be used in combination

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with classical transplantation approaches to follow cell fate in the periphery (Le Douarin, 1982). Moreover, when these genes were restricted to specific classes of neural cells, they become invaluable molecular markers, permitting researchers to unambiguously identify specific populations of neural progenitors, post-mitotic neurons and glia. Such markers can distinguish both between different classes of neural cells and different differentiation states within a class of neural cells, e.g. progenitor cells versus post-mitotic neurons. A further discovery that transformed developmental biology in the 1990s was the identification of fluorophores, first Green Fluorescent Protein (GFP) (Chalfie et al., 1994) and then a multitude of color variants (Giepmans et al., 2006), which permit researchers to label specific populations of cells with a fluorescent protein, supplied from a developmentally restricted promoter. Such genetically encoded fluorescent markers greatly simplify live imaging of cellular processes (Kaltschmidt et al., 2000). Thus, the existence of cell-type and differentiation-state specific markers has revolutionized our ability to follow developmental events in real time and determine the basis of neural identity and function. Here, we review what has been learned from these approaches in the PNS and CNS, focusing on the neural crest and developing spinal cord.

Origin of the peripheral nervous system and spinal cord

The vertebrate nervous system arises from the ectoderm, following induction of the neural plate in the gastrulating embryo. During the process of neurulation, the neural plate thickens and invaginates to form the cylindrical neural tube along the rostrocaudal axis of the embryo (Fig. 1A, B). In the head, the neural tube expands to form the brain, whereas it forms the spinal cord in the more caudal regions of the embryo (Fig. 1C). Shortly thereafter, neural crest markers become detectable in the dorsal-most portion of the newly closed neural tube along nearly the entire length of the body axis. Neural crest cells subsequently undergo an epithelial to mesenchymal transition, delaminate from the neural tube and commence migration to diverse and sometimes distant regions of the embryo (Le Douarin, 1982). Neural crest cells migrate into the periphery and contribute to the PNS, as well as many other derivatives (Fig. 1D), while the neural tube gives rise to the CNS.

At cranial levels, the peripheral nervous system has a dual origin from both cranial neural crest cells and ectodermal placodes (Baker and Bronner-Fraser, 2001; Couly and Le Douarin, 1985; D'Amico-Martel and Noden, 1983). The placodes give rise to cranial sensory ganglia and the sense organs (nose, ears, lens of eye); they are discrete regions of thickened columnar epithelium within the head ectoderm (Webb and Noden, 1993). Most placodes (otic, lateral line, epibranchial) form in the ectoderm adjacent to the neural tube except for the olfactory and adenohypophyseal placodes that originate within the anterior neural folds (Eagleson and Harris, 1990), the only region of the neural tube that does not form neural crest (Fig. 1C).

Further caudally, the entire PNS is derived from neural crest cells. “Vagal” neural crest cells arise from the neural tube just behind the ear and to the level to adjacent to somite 7. These cells migrate extremely long distances to form the enteric ganglia of the gut. This unique portion of the PNS is responsible for gut motility. At trunk levels, neural crest cells contribute to dorsal root and sympathetic ganglia of the PNS. The dorsal root ganglia are

sensory and form bilaterally adjacent to the developing spinal cord. They innervate the skin and various organs that sense proprioception, temperature and injury. Other neural crest cells migrate further ventrally to form the sympathetic chain ganglia. These cells innervate numerous organs along the length of the trunk.

At trunk levels, the remainder of the neural tube gives rise to the spinal cord. Initially only a single cell thick, the early neural tube is a pseudostratified epithelium comprised of rapidly dividing cells oriented perpendicular to the lumen of the tube (Fig. 1A) (Altman and Bayer, 1984). These cells are the progenitors for all of the neural and glial cells that will comprise the spinal cord. The neural tube first consists of two regions: an inner ventricular zone, containing the nuclei of the neural progenitors and the outer marginal layer, containing the processes of the neuroepithelial cells (Fig. 2A). The nuclei of the neural progenitors migrate in the perpendicular plane of the ventricular zone as a function of the cell cycle, such that mitosis takes place near the surface of the ventricle. As the progenitors begin to differentiate, the cells migrate laterally from the ventricular zone to form a third layer, the mantle layer, where the neural cell bodies reside. This layer will ultimately become the grey matter of the adult spinal cord. As development proceeds, the marginal layer expands to contain the fiber tracts, eventually becoming the white matter of the spinal cord (Fig. 2A).

Approaches for examining migratory pathways in the developing PNS

The methods used to examine the neural crest and placode contributions to various derivatives have evolved in sophistication over the years (Table 1). In particular, live imaging has greatly increased our understanding of the cell-cell interactions that occur during the migratory and differentiative processes. In the 1970s, Nicole LeDouarin made a great breakthrough enabling the analysis of neural crest migratory pathways and derivatives by devising quail-chick chimera assays in which quail neural tubes were grafted in place of chick neural tissue. These methods were later expanded to many other cell types including ectodermal placodes. Because quail cells can be followed long-term in the chick environment, this technique allowed for not only the mapping of neural crest migratory pathways but also the delineation of the full range of derivatives that formed from the neural crest in amniote embryos (Le Douarin, 1982). These studies and other cell labeling techniques have demonstrated that neural crest cells emigrate from the dorsal neural tube, migrate sometimes long distances, and give rise to a variety of derivatives at various axial levels. These classes include neurons and glia of sensory and autonomic ganglia, melanocytes, bone and cartilage, smooth muscle, chromaffin cells, and connective tissue of cranial muscles (Le Douarin, 1982) (Fig. 1D).

While quail-chick chimeras indelibly mark neural crest populations, this method requires surgical grafting between non-identical species, which may introduce artifacts. More recently, a number of methods for labeling populations of neural crest cells with vital fluorescent markers have emerged that do not require tissue grafting and offer the additional advantage of enabling live imaging (Table 1.). First, lipophilic dyes like DiI and DiO allow easy labeling of neural crest cells at distinct axial locations and times during their emigration process. In addition, they can be applied to many different vertebrates, allowing for comparative analysis across species. These labels have made it possible to follow the

progression of neural crest migration by time-lapse analyses in a number of species, including chick, frog and zebrafish. These studies have shown that neural crest cells fill their derivatives in a ventral to dorsal order and have revealed details of the migratory behavior (Kulesa et al., 2000; Raible and Eisen, 1996; Serbedzija et al., 1989). Second, electroporation and transgenesis techniques have emerged for labeling populations of neural crest cells with fluorescent tags like GFP or Red Fluorescent Protein (RFP) (Nie et al., 2011; Theveneau et al., 2010); RFP and fluorescent dextrans (Gross and Hanken, 2004) that have made possible high resolution confocal imaging (Kulesa et al., 2013; McKinney et al., 2013). These techniques have made it possible to study cell biological aspects of neural crest migration in many species, enabling the identification of ‘leaders’ and ‘followers’ and neural crest cell-cell interactions (Wynn et al., 2013) and examination of the developmental potential of neural crest cells at different times of exit from the neural tube (McKinney et al., 2013). Because these techniques unilaterally label the neural tube, they have revealed that contralaterally migrating neural crest cells are a major source of progenitor cells for the pain- and temperature-sensing afferents of the dorsal root ganglia (George et al., 2007). Vital dye labeling offers the advantage of marking cells without the need for generating interspecific grafts. Equally, GFP and fluorescent dextrans can be introduced into any species to perform intraspecific grafts (Gross and Hanken, 2004; Theveneau et al., 2010). However, dye labeling and electroporation are transient techniques that introduce dyes or genes for a few days, rather than indelibly labeling neural crest cells. Thus, they complement rather than replace previous interspecific grafting methodologies. The availability of transgenic zebrafish lines that label neural crest populations with Sox10 (Rodrigues et al., 2012) or FoxD3 (Hochgreb-Hagele and Bronner, 2013) circumvents this difficulty and enable long-term studies of neural crest migration and contributions to subsets of derivatives. Similarly, several mouse lines have utilized Cre recombination to label neural crest lineages under control of various promoters expressed in neural crest subpopulations. For example, the Pax3 lineage labels a population of melanocyte precursors, that then require repression of Pax3 to execute the full melanocytic program (Lang et al., 2005). The most famous of the Cre-based lineage tracing methods for neural crest utilize the Wnt1-cre line. However, many of the promoters used to study early neural crest formation control the expression of genes that are expressed only after neural crest induction, or are not specific to the neural crest cells. Importantly, the most widely used Cre line (Wnt1-Cre) recently has been shown to cause activation of Wnt signaling in the midbrain (Lewis et al., 2013). Thus, some studies using this line to lineage label neural crest cells may need to be reevaluated. Given that similar neural crest lineage contributions have been noted with other cre-driver lines (e.g. Pax3-cre, Pax7-cre) a as with the original Wnt1-cre line (Murdoch et al., 2012), this may not be a problem for all axial levels.

While all of these methods have different advantages and disadvantages, all reveal similar pathways of neural crest migration and derivatives. Importantly, similar approaches can be applied to both the neural crest and the spinal cord (see Table 1). Taking classical and modern methods together, these approaches have greatly increased our knowledge of the migration pathways and mechanisms underlying neural crest migration.

Interestingly, these studies have shown that there are different regional populations of neural crest cells along the body axis, designated as cranial, vagal, trunk and lumbosacral (Fig. 1C).

Cranial neural crest cells are the most diverse, as they contribute not only to cranial ganglia but also mesenchymal derivatives of the head, including cartilage, membranous bone, ocular tissues and melanocytes (Couly et al., 1993; Johnston et al., 1979; Le Douarin, 1982; Noden, 1975, 1978a, b, 1983a, b). The vagal neural crest emerges from the neural tube between the otic vesicle and the caudal boundary of the seventh somite; cells emerging from this level migrate into pharyngeal arches 3, 4, and 6 where they contribute to connective tissues, blood vessels, the cardiac outflow tract, parasympathetic innervation of the heart and the entire enteric nervous system that innervates the gut (Kirby et al., 1983; Le Lievre and Le Douarin, 1975). Within the gut, the earliest-generated crest cells move as a wave from anterior to posterior to populate the bowel. Trunk neural crest from the region caudal to the seventh somite gives rise to melanocytes, smooth muscle, sensory and autonomic neurons and glia, Schwann cells and adrenal chromaffin cells (Weston and Butler, 1966). Unlike cranial neural crest, trunk neural crest normally does not contribute to mesenchymal derivatives like smooth muscle, bone or cartilage. The very caudal (lumbosacral) portion of the neural tube also makes a small contribution to the enteric nervous system, primarily to glia.

Such fate mapping studies have suggested that the neurons of cranial sensory ganglia, such as the acoustic and epibranchial ganglia, are entirely derived from the placodes. Placodal derivatives include ciliated sensory receptors, sensory neurons, neuroendocrine and endocrine cells. However, neurons within the trigeminal ganglion have a dual origin from placodes and neural crest. Neurons of the olfactory epithelium and inner ear are primarily placodal in origin. One exception is the olfactory microvillous neurons of zebrafish, which are neural crest derived (Saxena et al., 2013). In contrast to the dual origin of cranial sensory neurons, lineage studies have shown that all of the glia of the peripheral nervous system, including olfactory ensheathing cells, originate from the neural crest in both chicken and mouse (Barraud et al., 2010; Forni et al., 2011; Le Douarin, 1982; Murdoch et al., 2010).

The basic anatomical features of the spinal cord

Distinct classes of neurons arise at different positions along the dorsal-ventral axis of the spinal cord (Fig. 2C). This organization of the spinal cord results in the different laminae of the spinal cord containing neurons segregated according to their distinct physiological properties and functions (Rexed, 1954). In general, cells associated with control of motor functions are located in or adjacent to the ventral horns whereas cells mediating sensory activities are present within the dorsal horn. In the ventral spinal cord, derived from the basal plate, motor neurons (MNs) send efferent projections out the ventral root to innervate the axial, hypaxial and limb musculature (Bonanomi and Pfaff, 2010), while ventral interneurons (INs) tend to make local excitatory and inhibitory regulatory circuits with MNs (Goulding, 2009). The motor nerves coalesce with the sensory nerves from the dorsal root ganglia at the rami to form the spinal nerves (Gallarda et al., 2008; Wang et al., 2014). The segmented nature of the dorsal root ganglia, results in a longitudinal array of efferent spinal nerves that innervate axial-specific targets in the periphery (Fig. 2G). In the dorsal spinal cord, derived from the alar plate, INs process afferent sensory input from the periphery or relay sensory information to higher order centers in the brain including the brainstem, thalamus and cerebellum (Bermingham et al., 2001; Braz et al., 2014). Sensory afferents from the dorsal root ganglion enter the spinal cord through the dorsal root entry zone and

terminate on INs in specific lamina in the dorsal horn. In turn, many populations of dorsal INs project axons commissurally, across the ventral midline into the ventral funiculus or ipsilaterally, into the dorsal lateral funiculus (Wentworth, 1984; Yaginuma et al., 1991). The intermediate spinal cord contains classes of INs that regulate sensory and motor functions, for example the dorsal INs that communicate with MNs to modulate the reflex-specific MN output required for the regulation of movement (Goulding, 2009). Towards the end of neurogenesis in the spinal cord, a period of gliogenesis occurs, resulting in the spatially restricted specification of astrocytes and oligodendrocytes (Hochstim et al., 2008; Muroyama et al., 2005; Rowitch and Kriegstein, 2010).

The molecular and cellular organization of spinal cord

Neural induction occurs from polarized mesodermal structures

The spinal cord is populated by multiple distinct populations of neurons and glia born in different locations and times along both the rostral-caudal and dorsal-ventral axes of the spinal cord (Ramón y Cajal, 1995). These patterns of differentiation are established by diffusible signals from the dorsal and ventral midline of the spinal cord and the surrounding paraxial mesoderm. These signals act on proliferating progenitor neurons and stem cells in the ventricular zone. After assuming their identity, neural progenitors exit the cell cycle, differentiate and migrate laterally into the mantle layer (Fig. 2A).

The process of gastrulation permits the neural plate to develop in concert with the surrounding mesodermal structures. These mesodermal structures include the paraxial (presomitic) mesoderm, which gives rise to the somites, and the notochord, which underlies a cluster of specialized neuroepithelial cells called the floor plate at the ventral midline of the spinal cord (Placzek et al., 1991) (Fig. 1B). Work performed in both chicken and mouse embryos has shown that the paraxial mesoderm influences several aspects of spinal development: in caudal regions, members of the Fibroblast Growth Factor (FGF) family permits spinal neural progenitors to remain proliferative (Diez del Corral et al., 2002); more rostrally, the presence of retinoic acid in somites directs spinal neuronal differentiation and establishment of dorsal-ventral patterning by promoting intermediate spinal cord fates (Diez del Corral et al., 2003; Novitch et al., 2003; Pierani et al., 1999). In the earliest inductive events within the spinal cord, the floor plate is induced by Sonic Hedgehog (Shh) present in the underlying notochord (Roelink et al., 1994) and the roof plate, a similar cluster of neuroepithelial cells, forms in the region where the neural plate fuses to make the neural tube (Fig. 1A, 2C) (Altman and Bayer, 1984). The roof plate is induced in response to signals from ectodermally-derived members of the Bone Morphogenetic Protein (BMP) family (Liem et al., 1995). These midline structures then themselves become critical organizing structures, secreting the inductive growth factors that pattern the neural identity of the surrounding tissue (Briscoe and Novitch, 2008; Le Dreau and Marti, 2012).

Neural identity along the dorsal-ventral axis

The discovery of molecular markers, most notably transcription factors whose mRNA expression or protein distribution can be identified though *in situ* hybridization experiments or immunohistochemistry respectively, has revolutionized our understanding of the cellular

organization and function of the embryonic spinal cord. Studies performed in both mouse and chicken embryos have demonstrated that neural progenitors arise in the ventricular zone, and are subdivided into discrete progenitor (p) domains, defined by the combinatorial code of their transcription factors (Fig. 2C) (Briscoe et al., 2000; Jessell, 2000; Shirasaki and Pfaff, 2002). The identity of the progenitor domains is dependent on signaling from the ventral and dorsal midlines as well as the paraxial mesoderm.

The notochord and floor plate in the ventral spinal cord produce a ventral-high to dorsal-low gradient of Sonic Hedgehog (Shh). Shh acts as a morphogen; the concentration and duration of Shh signaling is decoded by signaling machinery in the primary cilia on the apical side of progenitors in the ventricular zone (Briscoe and Ericson, 2001; Dessaud et al., 2007; Sasai and Briscoe, 2012). This process results in the formation of at least five progenitor domains in the ventral spinal cord: p0-3 and pMN (Fig. 2C). Sharp boundaries between the progenitor domains are then established by cross-repressive interactions between transcription factors present within the domains themselves (Alaynick et al., 2011; Briscoe et al., 2000; Muhr et al., 2001). Newly differentiated neurons exit the cell cycle, producing four major classes of ventral INs (v0-3) and the MNs. Post-mitotic neurons within a given domain, most notably the MNs, can diversify further (see below and also (Alaynick et al., 2011)). A number of modes of diversification have been identified including different migration paths (Sockanathan and Jessell, 1998), being derived from distinct subpopulations of neural progenitors within a domain (Agalliu et al., 2009), or through intercellular interactions as the neurons differentiate. For example, the division of the V2 INs, into the $V2_a$ and $V2_b$ subclasses, occurs through differential activation of the Notch signaling pathway (Del Barrio et al., 2007; Okigawa et al., 2014; Peng et al., 2007; Rocha et al., 2009).

In the dorsal spinal cord, there are seven known progenitor (dp) domains, the early born dp1 – dp6, and the later born dpIL, which together give rise to seven classes of dorsal INs (Fig. 2C; dI1- dI6, dIL) (Alaynick et al., 2011). As with the ventral spinal cord, codes of homeodomain and bHLH transcription factors first define, discrete progenitor domains and then distinct classes of post-mitotic neurons (Helms and Johnson, 2003; Zhuang and Sockanathan, 2006). The identity and proliferative capacity of the dorsal-most progenitor neurons (dI1 to dI3) is dependent on members of the BMP and Wnt families secreted by the roof plate (Liem et al., 1997; Megason and McMahon, 2002). The BMPs have been hypothesized to pattern the dorsal spinal cord by acting as morphogens (Lee and Jessell, 1999). However, this model was proposed largely by analogy with the patterning events in the ventral spinal cord. There are many BMPs present in the roof plate (Butler and Dodd, 2003; Liem et al., 1997) and studies have also suggested that some of them have specific effects on the induction of particular neural fates (Le Dreau et al., 2012; Lee et al., 1998). An additional four classes of INs (dI4 to dI6, dIL) arise independently from signals from the roof plate; their identity may be dependent on signals from the adjacent paraxial mesoderm (Le Dreau and Marti, 2013).

Neurogenesis generally occurs for only a brief time in embryogenesis, the progenitors then switch to give rise to distinct classes of glial cells (Rowitch and Kriegstein, 2010). MNs share a common ancestral progenitor with oligodendrocytes (Masahira et al., 2006; Sanes et

al., 1986; Sun et al., 2006); ventral INs share a common ancestor with the different subclasses of astrocytes that colonize different regions of the grey and white matter according to their site of origin (Hochstim et al., 2008; Muroyama et al., 2005; Rowitch and Kriegstein, 2010; Tsai et al., 2012).

Neural identity along the rostrocaudal axis

The rostral-caudal axis of the spinal cord forms progressively, such that the events of neurogenesis are spatially and temporally separated. The mouse spinal cord is most properly subdivided into five regions, based on the location of the cervical (encompassing the C1-8 segments), thoracic (T1-13), lumbar (L1-6), sacral (S1-4) and coccygeal (Co1-3) nerves (Fig 2E). However, researchers also often use a looser nomenclature taken from chicken embryogenesis to subdivide the spinal cord into five levels: the cervical (neck), brachial (forelimbs), thoracic (trunk), lumbar (hindlimb) and sacral (tail) levels.

Of the different classes of spinal neurons, the specific identities along the rostal-caudal axis have been most thoroughly assessed for MNs. MNs arise from the $Olig2^{+}$ pMN, and are arranged longitudinally into motor columns that innervate specific targets at different segmental levels (Davis-Dusenbery et al., 2014). As spinal MNs are generated, they segregate into different functional classes defined by their expression of specific codes of Hox-, Lim- and Forkhead homeodomain transcription factors, the positions of their cell bodies within the spinal cord and the patterns of their axonal trajectories (Fig. 2E). MNs in the medial motor column (MMC) are found along the entire axis of the spinal cord, where they innervate the axial musculature (Tsuchida et al., 1994). MNs in the hypaxial motor column (HMC) are found at most axial levels (cervical, thoracic and lumbar) where they are associated with the innervation of the muscles associated with respiration (Rousso et al., 2008). In contrast, MNs in the lateral motor column (LMC) are found only at limb levels and innervate distinct muscles in the limbs (Rousso et al., 2008; Tsuchida et al., 1994). There is topography to the LMC projection; MNs in the lateral and medial subdivisions of the LMC project axons towards dorsal and ventral limb muscles, respectively (Fig. 2F) (Landmesser, 1978a, b). At thoracic and sacral levels of the spinal cord, the preganglionic column (PGC) forms in the place of the LMC; PGC MNs innervate the sympathetic and parasympathetic nervous system (Markham and Vaughn, 1991; Prasad and Hollyday, 1991). The rostral-caudal identity of the different MNs columns depends in part on the mutual antagonism between the FGF and RA pathways, which regulates Hox gene expression (Dasen et al., 2003; Liu et al., 2001; Mazzoni et al., 2013). Rostral-caudal identity is then assigned by the expression of specific Hox genes to determine the segmental positional identities of neurons along the length of developing spinal cord. For example, the expression of Hoxc6/a6 directs the forelimb LMC MN identity, whereas Hoxc9/a9 directs PGC MN formation, and Hoxa10/d10 directs lumbar LMC MN fates (Fig. 2D) (Dasen and Jessell, 2009; Philippidou and Dasen, 2013).

Axon guidance mechanisms in the spinal cord

The past two decades has seen remarkable strides in our understanding of the mechanisms that establish spinal circuitry. Two of the most intensively studied trajectories are the dorsal commissural axons and the ventral motor axons:

Commissural neurons

Commissural neurons extend axons contralaterally thereby connecting the two sides of the spinal cord. Classic studies, dating back to Cajal, have shown that there are multiple classes of commissural axons along the dorsal-ventral axis of the spinal cord, all of which cross the spinal cord at the floor plate (Ramón y Cajal, 1995; Wentworth, 1984). However, the most attention has been directed towards understanding the trajectory of a specific class of commissural neurons, the dI1 INs, which differentiate immediately adjacent to the roof plate (Fig. 2B, C). dI1 axons are directed ventrally in response to members of the BMP family present in the roof plate here acting as a chemorepellents (Augsburger et al., 1999; Butler and Dodd, 2003; Yamauchi et al., 2008) to both orient and slow the rate of axon growth through the dorsal spinal cord (Perron and Dodd, 2011; Phan and Butler, 2013; Phan et al., 2010; Yamauchi et al., 2013). $Tag1^{+}$ dI1 axons extend towards the floor plate by taking a circumferential route through the transverse plane of the spinal cord (Fig 2B). dI1 axons have a tripartite temporal response to the floor plate (Dickson and Zou, 2010): first, they are attracted to it in response to Netrin1 and Shh, acting as long-range chemoattractants emanating from the floor plate (Charron et al., 2003; Kennedy et al., 1994; Serafini et al., 1996). Second, they cross the floor plate aided by local cell adhesive interactions provided by immunoglobulin domain superfamily members, such as Tag1/Axonin1 and NrCAM (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997). Finally, after crossing the floor plate, dI1 axons turn sharply orthogonally to join the ventral funiculus and project rostrally towards the brain (Bovolenta and Dodd, 1990; Kadison and Kaprielian, 2004). dI1 switch responsiveness to the floor plate by detecting the presence of multiple ventral chemorepellents, including the Slits and Semaphorins, through the modulation of receptor signaling in the dI1 growth cone (Long et al., 2004; Stein and Tessier-Lavigne, 2001). In mice, the sharp orthogonal turn is mediated by a rostral-high gradient of Wnt4 in the floor plate (Liu et al., 2005; Lyuksyutova et al., 2003), whereas a caudal-high Shh-signaling gradient may be important in chicken (Bourikas et al., 2005).

Motor neurons

Spinal MNs are essential for the movement of the limbs and trunk. As MNs are generated, they segregate into different functional classes that are defined by both the position of their cell bodies within the spinal cord and the pattern of their axonal projections, as described above. The mechanism underpinning the trajectory of the LMC motor axons towards muscles in the developing limbs has been studied most completely (Fig. 2E). The LMC MNs segregate further into distinct lateral and medial subcolumns, the LMC_l and LMC_m, which project axons towards dorsal and ventral limb muscles, respectively (Bonanomi and Pfaff, 2010) (Fig. 2F, G). MNs choose their particular axonal trajectory based on the “code” of LIM homeodomain (HD) and Forkhead domain proteins present (Bonanomi and Pfaff, 2010; Kao et al., 2012). Eph-ephrin signaling acts downstream of the Lim-HD protein code to

differentially regulate motor axon outgrowth into the limb at a region at the base of the limb called the plexus (Fig. 2F). Ephephrins work in a repulsive manner, such that, the presence of EphA4 on LMC_l axons directs them away from the repellent ligand ephrinA5 present in the ventral limb mesenchyme (Kania and Jessell, 2003). Similarly EphB receptors direct LMC_m axons away from ephrinB ligands in the dorsal limb (Luria et al., 2008).

Cell lineage studies in the developing peripheral and central nervous systems of the trunk

Neural crest cell lineage analysis

The diversity of neural crest derivatives has led to alternate proposals that suggest neural crest cells may already be determined to a particular fate prior to exiting the neural tube (unipotent) and/or may be naïve, multipotent, and influenced by extrinsic factors encountered during their migration. It is most likely that some combination of these states exists and influences neural crest cell fate decision. Numerous in vivo studies have demonstrated that there is plasticity in neural crest cell fate if premigratory neural crest cells are transplanted into different environments (Baker et al., 1997; Le Douarin, 1982; Nakamura and Ayer-le Lievre, 1982).

Single cell lineage analyses that test the developmental potential of individual neural crest either by injection of individual dorsal neural tube cells or migrating neural crest with a fluorescent dye (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988; Selleck and Bronner-Fraser, 1995) or with replication incompetent retroviruses (Frank and Sanes, 1991) have demonstrated that many neural crest precursors are multipotent. Similarly, clonal analyses in vitro have shown that neural crest cells are capable of forming multiple phenotypes (Dupin, 1984; Sieber-Blum and Cohen, 1980; Stemple and Anderson, 1993). These studies have demonstrated that clones derived from different axial levels can form numerous derivatives, and many/most clones give rise to multiple derivatives. These derivatives include various combinations of neurons and glia, adipocytes, melanocytes and cartilage (Baroffio et al., 1988; Dupin, 1984; Sieber-Blum and Cohen, 1980). These results support the idea that many individual neural crest cells are “multipotent”, having broad developmental potential that enables differentiation into several diverse derivatives. Occasionally but more rarely, clones that form a single cell type also have been observed. Although these cells are often referred to as “unipotent”, these conditions only test the normal fate of these clones, rather than their developmental potential, which is always greater than or equal to a cell’s normal fate. Thus, one can only draw meaningful conclusion about a cell’s developmental potential by challenging the original precursor under different experimental conditions.

Although many neural crest cells appear to be multipotent at the onset of migration (Bronner-Fraser and Fraser, 1988; Frank and Sanes, 1991), lineage restriction is thought to occur during migration and/or as their progeny reach their destinations. Neural crest can be induced to follow particular fates by culturing with specific growth factors; for example, they form neurons when cultured with BMP, generate glial cells with neuregulin (Lemke and Brockes, 1984; Shah et al., 1994) and differentiate into smooth muscle when cultured with

TGFβ (Anderson, 1997; Shah et al., 1996). Neurogenic genes such as neurogenins and Ascl (Mash1) appear to influence the type of neurons produced by neural crest cells (Anderson, 1993; Guillemot et al., 1993; Ma et al., 1998; Ma et al., 1999). Recent experiments have demonstrated that "neural crest stem cells", that are multipotent and retain the ability to contribute to neural crest derivatives, persist in many fetal and adult tissues, such as the peripheral nerve (Morrison et al., 1999) and skin (Jinno et al., 2010). For example, cell lineage analysis using GFP constructs introduced into the neural tube have demonstrated that many neural crest-derived melanocytes appear to originate from neural crest stem cells, likely to be Schwann cells or their precursors, that reside within the peripheral nerve (Adameyko et al., 2009).

In addition to single cell microinjection and clonal analysis in vitro, another method for neural crest cell lineage analysis is via the use of replication-incompetent retroviruses that can be applied at limiting dilutions with the goal of labeling single or small groups of cells in the central or peripheral nervous systems in vivo (Frank and Sanes, 1991; Price et al., 1987). In the chick, this retroviral labeling has successfully been applied to cell lineage analyses of both neurons and glia in dorsal root and enteric ganglia, spinal cord, retina and optic tectum (Frank and Sanes, 1991; Galileo et al., 1992; Gray et al., 1988; Leber et al., 1990; Mikawa et al., 1991). This method is not only simple but also allows progeny of labeled cells to be followed throughout development, since the marker is integrated into the genome. In addition, introduction of virus is much easier than injection of intracellular dye or tracer. Because viruses can now be used to introduce multiple colors simultaneously it is possible to follow complex lineage relationships in individual embryos. For example, the "brainbow" technique utilizes random expression of different color variants of GFP to distinguish lineally related cells by virtue of the ratios of the various red, green and blue fluorescent proteins, such that only those sharing a lineage express the same color (Livet et al., 2007). Using this approach, it is now possible to follow multiple clones within a single embryo (Cai et al., 2013). This method has been successfully applied to a range of vertebrates from mice to zebrafish.

Recently, these types of approaches in mice have demonstrated that much of the parasympathetic system arises from neural crest-derived glial cells, which appear to be Schwann cell precursors that are resident in nerves (Dyachuk et al., 2014; Espinosa-Medina et al., 2014). These bi-potent progenitors appear to be able to generate both glia and neurons. In addition, the precursors appear to contribute to melanocytes (Adameyko et al., 2009) and thus may represent a type of neural crest stem cell that remains resident along peripheral nerves.

Lineage analysis in the developing spinal cord

In the early 1990s, Thomas Edlund and colleagues found that antibodies directed against the Lim-homeodomain transcription factor Islet1, specifically labeled post-mitotic spinal MNs (Ericson et al., 1992). Over the next two decades, the use of *in situ* hybridization in combination with antibodies generated against a variety of transcription factors, including basic Helix-loop-helix (bHLH), Forkhead-, Lim-, Paired- and Pou-homeodomain transcription factors, became the pre-dominant method by which cell fate/lineage is assigned

in the spinal cord (Alaynick et al., 2011) and revolutionized our understanding of both the organization of the spinal cord, and the mechanisms that establish spinal cell fates (Briscoe and Novitch, 2008). There are some limitations to this observational technique: the dynamic nature of transcription factor expression makes it challenging to follow migrating populations of neurons, and given that transcriptional factor expression is generally not sustained in specific populations, it is hard to follow the fate of neural populations over extended time (Table 1.)

In general, transcription factors are present in overlapping subsets of cells along the dorsal-ventral axis such that any given progenitor domain is defined by a code of one to >3 transcription factors (Fig. 2B, C). This transcription factor code endows progenitors with distinct functional characteristics that influence their proliferative capacity, timing of differentiation, and the type of cells that they will ultimately produce. As cells within the progenitor domains mature, the complement of transcription factors expressed by the progenitors frequently directs the code of transcription factors expressed by the post mitotic neurons. This phenomenon is observed in both the dorsal and ventral spinal cord. For example, the bHLH transcription factor Atoh1 (Math1) both labels the pd1 cells and is required for their identity (Gowan et al., 2001; Helms and Johnson, 1998). As these dorsal progenitors differentiate, the post-mitotic dI1s then express the Lim-homeodomain transcription factors Lhx2/9 (Zhuang and Sockanathan, 2006). Another canonical example is found in the ventral spinal cord, where MN progenitors are distinguished by the highly specific expression of the bHLH transcription factor, Olig2 (Fig. 2B) (Novitch et al., 2001). $Olig2^+$ progenitors differentiate into $Isl1/2^+$ MNs (Briscoe and Novitch, 2008; Pfaff et al., 1996). However, the pMN cells are multi-potential: retroviral tracing has shown that these cells produce both MNs and oligodendrocyes (Leber et al., 1990) and indeed the genetic ablation of Olig2 results in the complete loss of both cell types (Lu et al., 2002). In addition to mouse genetic models, the mechanisms that assign cell fate in the spinal cord have been principally examined using *in vitro* tissue culture assays, or by manipulating gene expression by the *in ovo* electroporation of chicken embryos. A particularly successful strategy has been to assess cell fate within *in vitro* explants of intermediate spinal cord, i.e. the region equidistant from the dorsal and ventral midlines, taken from chicken embryos at or before the neural fold stage (Yamada et al., 1993). At this early stage, the presumptive intermediate neural tube is developmentally naïve, yet to receive dorsalizing or ventralizing signals. These explants have thus been an ideal canvas on which to examine the role of extrinsic patterning signals and have been used extensively to examine the mechanistic basis of Shh signaling (Ericson et al., 1997), with the caveat that such explants are obviously removed from their endogenous context.

The use of dye filling or viral infection, the classic means of tracking cell lineage over time, has been relatively limited in the developing spinal cord after the identification of molecular markers. Nonetheless they remain the definitive methods for identifying the progeny of a single population of cells. For example, retroviral tracing in the dorsal spinal cord has shown that progenitors in dIL domain can divide asymmetrically to produce both dIL_A and dIL_B post-mitotic neurons (Wildner et al., 2006). Moreover, dye filling, most notably with DiI or DiO, remains a critical, albeit non-specific method for following neural processes in the spinal cord (Bovolenta and Dodd, 1990; Kadison and Kaprielian, 2004), since few

antibodies have been identified that are specific to different classes of spinal axons (Dodd et al., 1988). Viral vectors, derived from adenoassociated or rabies viruses, also offer the promise of categorizing specific neural circuits established by the different classes of embryonic spinal neurons. These viruses can be genetically targeted to specific classes of spinal neurons, by using the enhancer regions of developmentally relevant genes, such as transcription factors described above with restricted expression in the spinal cord, to direct the expression of viral receptors to specific classes of neurons (Wickersham et al., 2007). The use of developmental restricted enhancers, where suitable examples are available, has also been invaluable for directing the expression of fluorescent proteins epitope tagged with either membrane or axonal localization signals, such as tau (Mombaerts et al., 1996), to specific axonal pathways in the mouse spinal cord (Bai et al., 2011; Imondi et al., 2007). Examples in the spinal cord include the Math1 enhancer, which drives the expression of markers specifically in dI1 commissural axons (Hazen et al., 2012; Helms and Johnson, 1998; Phan et al., 2010) and the Hb9 enhancer, which can drive the expression of heterologous genes in spinal MNs (Arber et al., 1999) and is invaluable for dissecting the branching patterns of motor nerves in the limb (De Marco Garcia and Jessell, 2008; Rousso et al., 2008). Electroporation-based strategies with developmentally restricted enhancers have also been developed to follow axonal pathways in the chicken spinal cord (Avraham et al., 2009; Phan et al., 2010). Similar methods in rodents have been more difficult to achieve given the technical challenges of targeting the spinal cord at early stages of development by *in utero* electroporation.

A more recent strategy to trace lineages in the developing spinal cord is to use inducible Cre-loxP fate mapping. Mice are engineered to express Cre recombinase from developmentally restricted enhancers, such that Cre is present only in specific cell types (Battiste et al., 2007). These mice are then crossed to a transgenic line containing a loxP-flanked transcriptional stop cassette in front of a reporter gene, usually *lacZ*, EGFP, or EYFP driven by a constitutively active genomic locus, for example the *Rosa26* locus (Srinivas et al., 2001). This reporter will be expressed in any spinal neural progenitors or post-mitotic neurons where Cre has been present. If the reporter gene produces a long-lived protein, such as β-galactosidase, this strategy results in an aggregated readout of the cell types that have expressed the chosen enhancer, although care is needed to distinguish between real verses random fluctuations in Cre expression. Cre lineage mapping in the spinal cord has been performed for many enhancers, including the Ngn1 enhancer, which drives expression in pd2 (Quinones et al., 2010), Ascl1/Mash1 enhancer (pd3, pd5, pdIL, (Battiste et al., 2007)), Dbx1 enhancer (pd6, p0, (Dyck et al., 2012)), Olig2 enhancer (pMN, (Dessaud et al., 2007)), and the Nkx2.2 enhancer (p3 cells, (Balderes et al., 2013)). These studies have revealed the range of cell types derived from these domains as well as providing important mechanistic insights into cell fate specification. For example, use of the Olig2::*cre* line provided compelling evidence that ventral neural progenitors interpret both the duration and concentration of Shh signals from the floor plate to direct ventral progenitors towards their ultimate cell fates (Dessaud et al., 2007). Methods of lineage tracing are continuously evolving; for example, the recently described multiaddressable genome-integrative color (MAGIC) method builds on the “Brainbow” method to trace the lineage of multiple neural progenitors simultaneously (Loulier et al., 2014).

Future directions

In the developing peripheral nervous system, there are gaps in our knowledge regarding the manner and timing by which cell lineage decisions are made during the course of neural crest and placode migration and condensation into peripheral structures. Information is accumulating regarding the gene regulatory events controlling the neural crest cell specification (Betancur et al., 2010). Similarly, much is known about transcriptional regulation regarding choice of neuronal and glia fate in various ganglia. However, less is known about interactions between progenitor cells and how their environment in turn influences determination of cell fate.

An important direction for future studies will be to visualize the process by which peripheral nervous system precursors move from their site of origin to their final sites in the developing PNS. With the advent of high-resolution microscopes and fluorophores of many colors, it will be possible to visualize many cell types simultaneously in living tissues. Imaging technologies like two photon light sheet microscopy have advanced enormously, increasing not only resolution but also depth of focus into intact tissue. These advances make it possible to view cells not only in tissue slices but also in intact organisms. Model systems like zebrafish, with its excellent genetics plus transparent embryo and larvae, will nicely complement studies done in amniotes, which more closely resemble humans. By accompanying imaging with perturbation analysis, much can be learned about cell lineage decisions in the intact organism. Novel methods for manipulating gene expression, like TALENS and CRISPR, for perturbation and genome editing will be extremely useful ways to alter cell lineage programs that can be applied to analysis of cell lineage decisions in the developing nervous systems as well as to reprogramming and directed differentiation of PNS and CNS cells for regeneration and repair.

The cellular and molecular organization of the developing spinal cord has been well described, particularly along the dorsal ventral axis, leading to the spinal cord being one of better-understood systems in the developing vertebrate nervous system. However there are still some notable gaps, for example the categorization of neural circuits generated by the different classes of spinal neurons remains incomplete. An atlas of embryonic spinal circuits would greatly assist in efforts to understand congenital abnormalities in spinal circuit formation. The extent to which the cell lineages identified in the avian and rodent spinal cord are conserved in human also remains unclear. However, recent advances in human stem-cell derived organoid cultures (Sasai, 2013) provide a unique opportunity to model human brain and spinal cord development in both control and disease states. Finally, the connection between the formation of the embryonic populations of spinal neurons and those in the mature spinal cord is not well understood. To what extent does molecular identity translate into function? Again, the emerging new methods of lineage and circuit tracing, gene perturbation and *in vivo* imaging are likely to shed light on these questions.

Taken together, these rapidly emerging novel technologies and improvements in both image analysis and genome manipulation are likely to transform our understanding of how the developing nervous system forms.

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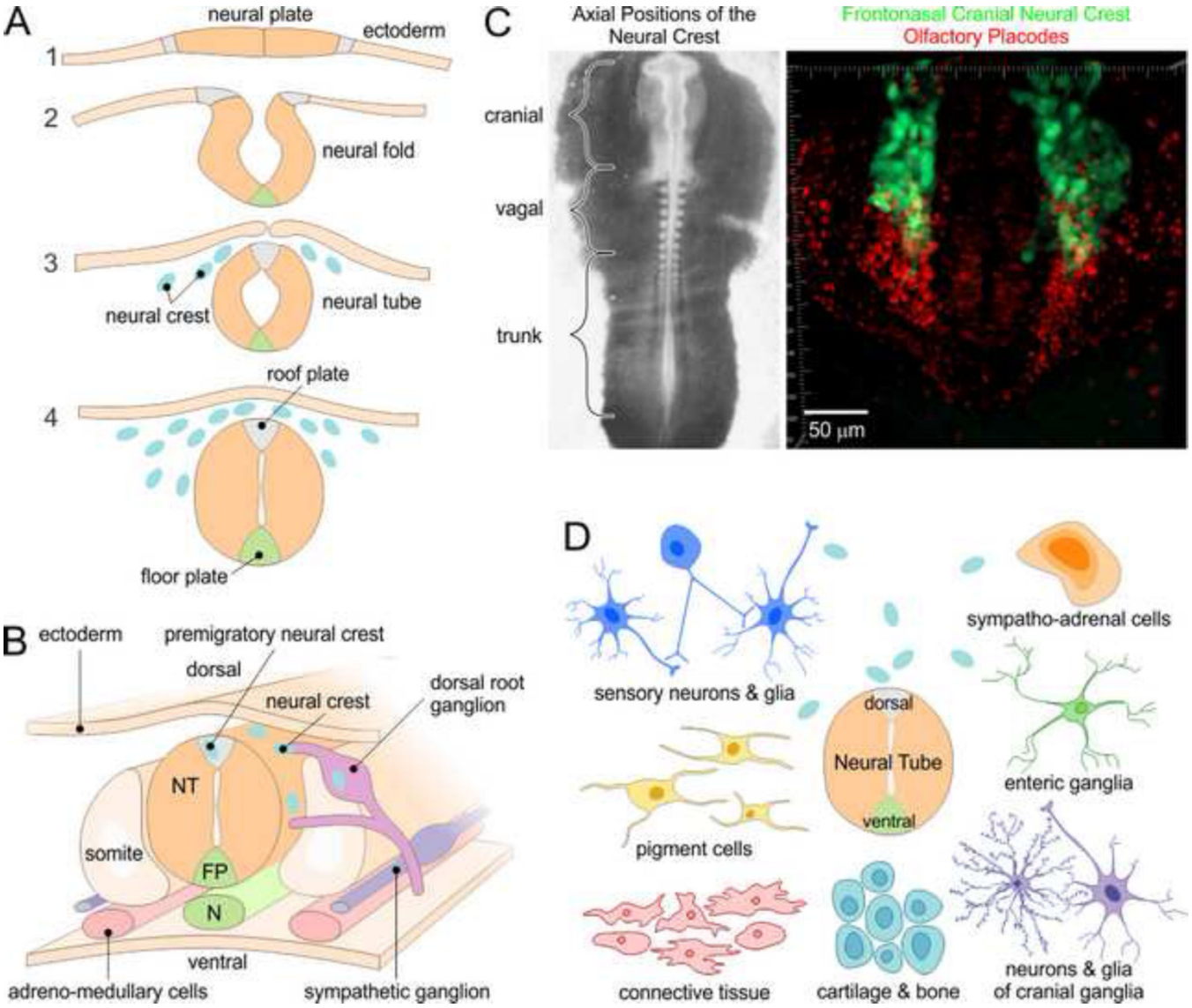

Figure 1. Formation of the spinal cord and peripheral nervous system

a) Schematic diagram illustrating the process of neurulation and onset of neural crest migration. 1. Initially, the ectoderm is open and flat. The neural plate thickens in comparison to the adjacent non-neural ectoderm. 2. During neurulation, the neural plate bends and begins to close. 3. Shortly after neural tube closure, neural crest cells emigrate from the dorsal portion of the neural tube and 4. continue migrating into the adjacent mesenchyme.. b) With time, neural crest cells condense to form multiple derivatives, including dorsal root ganglia, sympathetic ganglia, adrenomedullary cells

c) Different populations of neural crest cells arise from different axial levels of the chicken neural tube. Indicated in this whole mount view of an embryo are the relative sites of emergence of cranial, vagal, trunk and lumbosacral (further caudal but not shown here) neural crest cells. In the adjacent section, cranial neural crest cells expressing Sox10 intermingle with olfactory placode cells in a zebrafish embryo. Both will differentiate into olfactory sensory neurons within the olfactory epithelium.

d) Schematic diagram illustrating some of the diverse derivatives that arise from the neural crest, including PNS neurons and glia, pigment cells, and craniofacial cartilage.

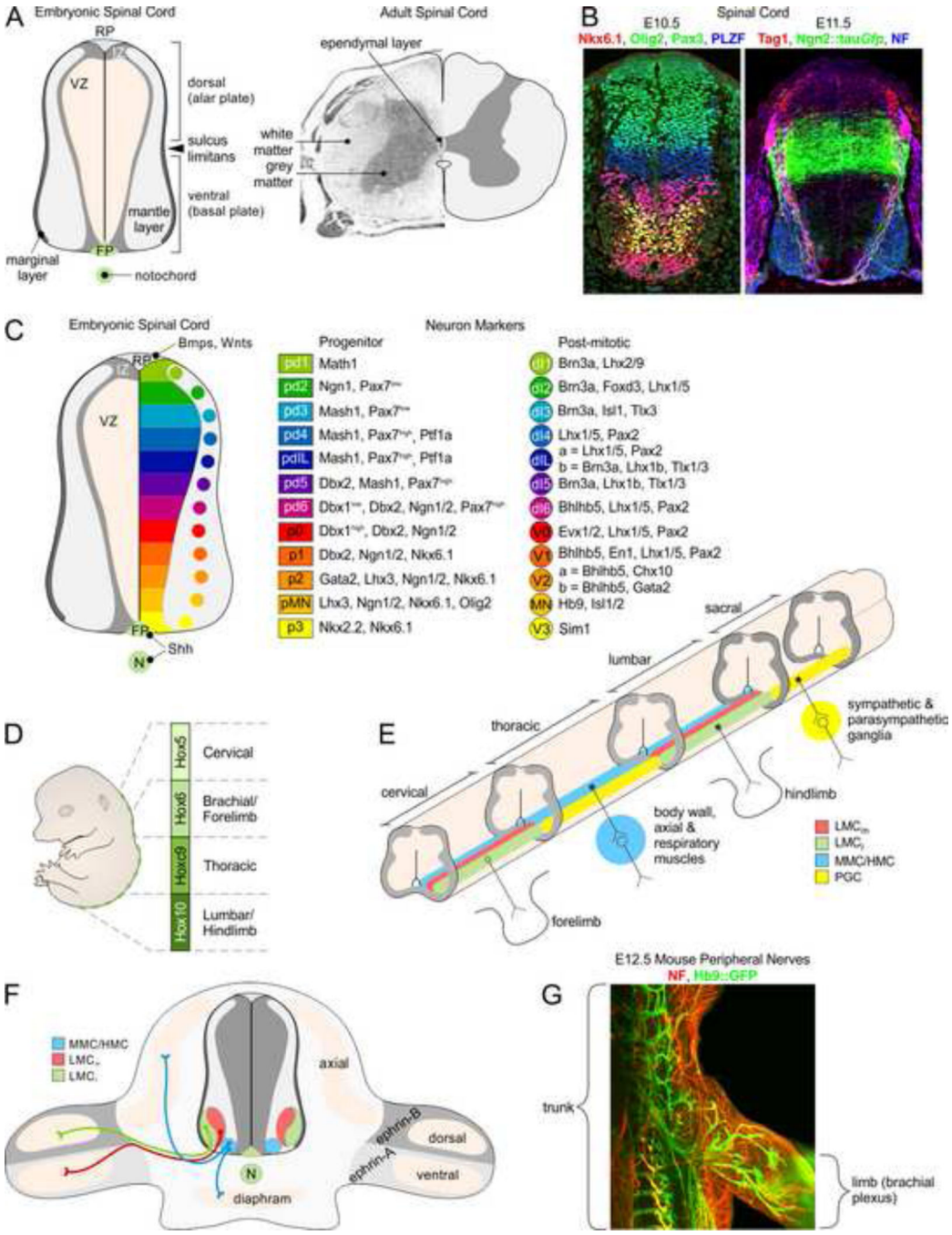

Figure 2. Spinal cord development

a) Cellular organization of the embryonic and adult spinal cord. The dorsal and ventral halves of the spinal cord are separated by the sulcans limitans (arrowhead). Neuronal progenitors proliferate in the ventricular zone, as they differentiate, they move laterally into the intermediate zone and then settle in the mantle layer, sending processes into the marginal layer. The mantle and marginal layers expand over time to become the grey and white matter in the mature spinal cord.

b) Organization of the spinal cord along the dorsal-ventral axis. The transverse section of the E10.5 spinal cord has been labeled with antibodies against Nkx6.1 (red), Olig2 (green), Pax3 (green) and PLZF (blue) to reveal the sharp boundaries between progenitor domains. In the section of E11.5 spinal cord, two populations of commissural axons are labeled with antibodies against Tag1 (red) and a genetically encoded transgene, Ngn2::tau*Gfp* (green). The complete complement of axons is labeled with antibodies against neurofilament (blue).
c) Distinct classes of neuronal progenitors in the ventricular zone, which give rise to distinct populations of postmitotic neurons. These progenitors and neurons can be distinguish by their unique complements of transcription factors, a subset of which are shown here (Alaynick et al., 2011).
d) The Hox code specifies the axial identity along the rostral-caudal axis of the spinal cord (Dasen and Jessell, 2009).
e) Organization of the motor columns along the rostral-caudal axis.
f) Summary of spinal motor axon connections at limb levels. The MMC motor axons innervate the axial musculature, the HMC motor axons innervate the respiratory muscles and the LMC motor nerves innervate the dorsal (LMC_l) and ventral (LMC_m) limbs, respectively.
g) Spinal nerves innervating the trunk and limb musculature. In this whole mount preparation of E12.5 Hb9::*Gfp* transgenic mouse at the brachial plexus, the GFP^+ motor nerves (green) and neurofilament$^+$ sensory nerves (red) are present in a segmental array that innervate axial-specific targets in the trunk and forelimbs.

Table 1

Technique for lineage tracing	Applied to neural crest studies?	Applied to spinal cord studies?	Key advantage for lineage tracing	Disadvantage for lineage tracing
1. Injection				
a. Lipophilic dyes (e.g. DiI or DiO)	yes	axon tracing	simple, *in vivo* live imaging possible	dye diluted with cell division
b. Replication incompetent viruses	yes	limited use	trace lineage of single cells	multiple markers required to assess clonality
c. Fluorescent proteins/ polysaccarides (e.g. lysinated fluorescein or rhodamine dextran, horseradish peroxidase, cholera toxin B-conjugates	yes	yes	injection of single cells assures clonality, retrograde and anterograde tracing of axon trajectories	label diluted with cell division
d. Electroporation of genes encoding fluorescent proteins (e.g. GFP, RFP, tdTomato)	yes	yes	simple technique for transient labeling	difficult to follow single cells
2. Observation				
a. In situ hybridisation	yes	yes	simple, identify site of gene transcription	static i.e. performed on fixed tissue, signal strength depends on mRNA copy number
b. Immunohistochemistry	yes	yes	simple, identify site of gene translation	challenging to follow motile processes, temporally limited to period that protein is present
c. Live imaging	cell migration	axon tracing	follow motile processes in real time	long-term studies challenging, must be able to visually identify cell of interest
3. Transplantation				
a. Xenografts (e.g. Chick-quail chimeras)	yes	no	permanent labeling	grafting errors, possible species differences
b. Tissue explants	yes	yes	bath application of extrinstic factors	tissue removed from endogenous context
4. Developmentally restricted enhancers				
a. Electroporation	yes	yes	*in vivo* labeling of specific cells	transient, challenging to perform *in utero*
b. Transgenesis (e.g. Lox-cre mice)	yes	yes	cumulative readout of gene expression	suitable enchancer must be available

Review of classical and modern lineage and marker analyses in the CNS and PNS
Neural crest cell lineage analysis reveals migratory pathways and multipotency
Spinal cord lineage tracing reveals regional organization of neurons and progenitors